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Abbreviations:

AM - alveolar macrophage, BAL - bronchoalveolar lavage, ELISA - enzyme-linked immunoassays, GM-CSF - granulocyte/macrophage-colony stimulating factor, iNOS - inducible nitric oxide synthase, ICAM-1 - intercellular adhesion molecule-1, IL – interleukin, MIP-2 - macrophage inflammatory protein-2, MCP-1 - monocyte chemoattractant protein-1, PMN – polymorphonuclear neutrophils, TGF -transforming growth factor, TNF-α - tumor necrosis factor-α, type II cells - alveolar epithelial type II cells.

Abstract

The expression of 10 genes implicated in regulation of the inflammatory processes in the lung was studied following exposure of alveolar macrophages (AM) to silica *in vitro* or *in vivo*. Exposure of AM to silica *in vitro* upregulated the mRNA levels of three genes (IL-6, MCP-1, and MIP-2) without a concomitant increase in the protein levels. AM isolated following intratracheal instillation of silica upregulated mRNA levels of four additional genes (GM-CSF, IL-1β, IL-10 and iNOS). Protein levels IL-6, MCP-1 and MIP-2 were elevated in bronchoalveolar lavage (BAL) fluid. Fibroblasts under basal culture conditions express much higher levels of IL-6 and GM-CSF compared to AM. Co-culture of AM and alveolar type II cells, or co-culture of AM and lung fibroblasts, in contact cultures or Transwell chambers, revealed no synergistic effect. Therefore, such interaction does not explain the effects seen *in vivo*. In conclusion, identification of the intercellular communication *in vivo* is still unresolved. However, fibroblasts appear to be an important source of inflammatory mediators in the lung.

Introduction

Occupational exposure to crystalline silica is associated with the development of pulmonary silicosis (Hnizdo and Vallyathan 2003; Reiser and Last 1979)and an increased risk for lung cancer (McDonald and McDonald 1995). Silica can cause direct DNA damage and mammalian cell transformation (Daniel et al. 1995; Shi et al. 1994). The initial event, however, is an inflammatory response, including oxidant production and recruitment of inflammatory cells into the lung.

A number of inflammatory mediators have been implicated in silica-induced pathology. Among them are included cytokines, such as interleukin-1β (IL-1β)(Goodman et al. 2003), IL-6 (Gosset et al. 1991), IL-10 (Huaux et al. 1998), tumor necrosis factor-α (TNF-α)(Dubois et al. 1989), and transforming growth factor (TGF)(Williams et al. 1993; Williams and Saffiotti 1995); chemokines, such as monocyte chemoattractant protein-1 (MCP-1)(Barrett et al. 1999) and macrophage inflammatory protein-2 (MIP-2)(Driscoll et al. 1993); the non-protein inflammatory mediator nitric oxide, generated mainly through inducible nitric oxide synthase (iNOS)(Castranova et al. 1998); and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1)(Hubbard and Giardina 2000; Nario and Hubbard 1996). However, the changes in the inflammatory mediators have been studied in different contexts. Some were studied *in vitro*, some *in vivo*, some in cell lines, and some in primary cells making it difficult to draw conclusions as to which of these mediators are involved in the initial phase of the lung inflammatory response and which become important in later stages of the response.

A second aspect that has not received much attention is the source of these inflammatory mediators and the importance of cell/cell interactions in the production of these mediators.

In our studies of silica-induced production of inflammatory mediators in alveolar macrophages (AM), we found the responses of AM following *in vitro* stimulation were quite different compared to AM isolated following *in vivo* exposure to silica. This suggests that cell/cell interactions may a play an important role in silica-induced production of inflammatory mediators in the lung. Previous studies have indicated a role for interaction between alveolar epithelial type II cells (type II cells) and AM in the production of iNOS (Pechkovsky et al. 2002) and a role for interaction between fibroblasts and AM in the production of granulocyte/macrophage-colony stimulating factor (GM-CSF; Fitzgerald et al. 2003).

The recent technical advances in PCR methodology make it possible to study the expression of several genes simultaneously even with small amounts of RNA. Therefore, to understand the role of inflammatory mediators in silica-induced pathology, we studied the expression of several inflammatory mediators in AM following *in vitro* exposure to silica or after *in vivo* exposure by intratracheal instillation. The expression was studied at the message level by real-time RT/PCR and, where appropriate, at the protein level by enzyme-linked immunoassays (ELISA). In addition, we studied the interactions between AM and type II cells or fibroblasts in *in vitro* culture systems. Our studies indicate that

the lung fibroblasts are an important source of inflammatory mediators following silica exposure.

Materials and Methods

Animals: The animals used in these experiments were specific pathogen-free Sprague-Dawley rats [HLA:(SD)CVF], (Hilltop Laboratories, Scottdale, PA), weighing 250-300 g (approximately 8 weeks old at arrival). The animals were housed in an AAALAC – accredited, environmentally controlled facility. The rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasmas, Helicobacter and CAR Bacillus. Rats were acclimated for at least 5 days before use and were housed in ventilated cages, which were provided with HEPA-filtered air, and used Alpha-Dri virgin cellulose chips and hardwood Beta chips as bedding. The rats were maintained on ProLaB 3500 diet and tap water, both of which were provided *ad libitum.*

Reagents: Rat cytokine kits for IL-6, MCP-1, MIP-2, and TNF-α were obtained from Biosource (Camarillo, CA). LDH was measured within 24 h on refrigerated samples with a COBAS MIRA® Plus (Roche Diagnostics, Indianapolis, IN) using kits from Roche. Lipopolysaccharide B (LPS; from E.*coli* 026:B6) was obtained from Difco Laboratories (Detroit, MI). The culture medium consisted of Dulbecco's modified Eagle medium (DMEM: Bio-Whittaker, Walkersville, MD), 1 mM glutamine (Sigma, St. Louis, MO), 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES; Sigma, St. Louis, MO), 100 U/ml penicillin-streptomycin (GIBCO), 100 μg/ml kanamycin

(GIBCO, Life Technologies, Grand Island, NY), and 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO).

Source of Silica: Min-U-Sil 5 was from US Silica (Berkeley Springs, WV). It was examined by proton-induced X-ray emission (PIXE) spectrometry for inorganic contaminants and for desorbable organic compounds by gas chromatography mass spectroscopy. The results of these analyses have been reported elsewhere (Porter et al., 2001). Silica samples were found to be >99% pure quartz. Mean particle count diameter, determined by scanning electron microscopy was 2.14 μm, with 99% of the particles being smaller than 5 μm. Silica was weighed and dry-heated at 170° C for 24 hours to sterilize. Sterile media was then added to the silica, vortexed into suspension prior to adding to the cell culture.

Isolation of Alveolar Macrophages: The animals were anesthetized with pentobarbital sodium (150 mg/kg body wt) and exsanguinated by cutting the abdominal aorta. AM were obtained by BAL according to the method of Myrvik et al. (1961). The lungs from each animal were lavaged eight times with 5 ml of phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.4 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4)/g lung weight. The cells were separated from the lavage fluid by centrifugation at 300 g for 5 min and then washed three times by alternate centrifugation and resuspension in phosphate-buffered medium. The cells were then resuspended in the culture medium for use in all experiments. Cell number was determined by an electronic cell counter (model ZB, Coulter Electronics, Hialeah, FL).

Isolation of Type II cells: Type II cells were isolated as described previously (Miles et al., 1997). Briefly, the procedure involves perfusing the lung to remove blood, removing free AM by BAL, digestion of lung tissue with elastase, and purification of type II cells by centrifugal elutriation. Cells isolated and purified by this method were greater than ≥ 85% pure type II cells as determined microscopically after staining with phopshine 3R (Jones et al. 1982).

The cells were cultured on collagen gels similar to those described by Lee et al. for growing hamster tracheal epithelial cells (Lee et al. 1984). Collagen gels were prepared from stock solution of Collagen Type I from rat tail (Sigma-Aldrich, St. Louis, MO) dissolved in 1:1000 dilution of acetic acid in sterile distilled water overnight at 4^{o}C. A 6-well plate was layered with 0.775 ml (each well) of ice-cold collagen gel mixture consisting of 0.5 ml of collagen stock, 0.15 ml of 10x MEM medium, and 0.125 ml of 0.5 N NaOH. The mixture was allowed to polymerize for a 4 h at a humidified atmosphere of 5% CO_2 at 37^{o}C. The polymerized collagen gels were washed with one ml of epithelial cell growth medium before cells were plated and grown overnight.

Isolation of Lung Fibroblasts: Lung fibroblasts were isolated as described by Reist et al. (1991). Briefly, the lungs were perfused with normal saline and lavaged with PBS containing 0.1% glucose and sectioned 4 times at 0.5 mm intervals with a McIlwain tissue chopper. The chopped lung tissue from a single rat was digested in 20 ml of HEPES-buffered solution (145 mM NaCl, 5 mM KCl, 1 mM CaCl2, 505 mM glucose, and 10 mM HEPES, pH 7.4), containing collagenase (0.1%), elastase (40 U/ml), BSA (0.5%) and DNAse (0.018%) in a shaker water bath for 30 min at 37° C. The digested mixture was filtered through 2 layers of sterile gauze that has been washed with culture medium. The cells were sedimented by centrifugation and plated in 6 well culture plates. The medium was changed 24 h later, and the cells were allowed to grow to confluence.

Co-Culture of Type II Cells and AM: Type II cells cultured overnight on collagen gels in 6 wells plates (Catalog # 353046; Tissue culture treated by vacuum gas plasma, polystyrene, nonpyrogenic, Becton Dickinson, Franklin Lakes, NJ), as described above, were incubated for an additional 4 hours at 37°C in a CO_2 incubator with freshly isolated AM (one million cells) with or without silica. Controls were type II cells alone with or without silica. The collagen gels were dissolved in a solution containing 1mg SIGMA Blend Collagenase Type F made up in 1ml of type II cell growth medium for each well to be dissolved. The cells were then spun down and used for isolation of total RNA.

Co-Culture of Lung Fibroblasts and AM: Lung fibroblasts were cultured until they became confluent. The cells were trypsinized and 2 $X10^6$ cells were plated in 6 well plates. After overnight culture, freshly isolated AM (2 $X10^6$ cells) were added to the wells and cultured for an additional 4 h with or without silica. Controls were fibroblasts alone with or without silica. The culture medium was aspirated, spun down, and the supernatant was stored at -80ºC. The cells were scraped and combined with the cell pellet from the above step and used for isolation of total RNA.

Transwell Experiments with Fibroblasts and AM: In order to measure mRNA expression in separated cell populations and to study the interaction of soluble mediators released by cell populations on each other, experiments were conducted in Transwell chambers (CoStar, Corning, NY). For these experiments, cultured lung fibroblasts were trypsinized and 1 million cells were plated in the outer well of Transwell plate and cultured for an additional 24 h. At the end of the 24 h period, freshly isolated AM (1 million cells) were placed in the inserts. Silica was added either to the macrophages in the inner wells or to

the fibroblasts in the outer well and incubated for 4 h. Total RNA was isolated from each population separately.

Preparation of AM- and PMN-Enriched Fractions: AM and PMN enriched fractions were obtained from BAL fluid obtained from rats treated with silica *in vivo*, as described by Huffman et al. (2003). Briefly, the method consisted of layering BAL cell populations obtained by lavage onto a Histopaque double-density gradient composed of equal amounts of Histopaque 1083 and Histopaque 1119 (Simga, St, Louis, MO). The gradients were then centrifuged (400 g, 30 min, RT). The AM-enriched fraction localized at the interface between PBS diluent and Histopaque 1083, and the PMN-enriched fraction was located at the bottom as a pellet. This method yields about 60% AM in AM-enriched fraction and 90% PMN in PMN-enriched fraction (Huffman et al., 2003).

Measurement of Cytokines: The cytokines were measured in culture supernatants after a 24 h incubation with either 200 μg/ml silica or 1 μg/ml of LPS. IL-6, MCP-1, MIP-2 and TNF-α were measured by ELISA kits according to manufacturer's instructions (Biosource International, Camarillo, CA). The values were expressed as nanograms or picograms/million cells. For measurement of cytokines in the BAL fluid, lavage fluid from the first wash was collected and spun down to sediment the cellular elements. The supernatant was stored at -80^{o}C for later measurement of cytokine levels by ELISA.

Quantitation of mRNAs by RT/PCR: We measured cytokine mRNA levels using a SYBR Green PCR kit with the ABI 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Total RNA was isolated using RNAqueous™ -4PCR kits (Ambion, Austin, TX) from AM (≈ 2 million cells) or lung tissue following alveolar lavage (≈ 50 mg of wet

tissue). One to two micrograms of the DNAse I-treated RNA was reverse transcribed, using Superscript II (Life Technologies, Gaithesburg, MD). The cDNA generated was diluted 1:100 and 15 μl was used to conduct the PCR reaction according to the SYBR Green PCR kit instructions. The Comparative C_T (threshold cycle) method was used to calculate the relative concentrations (User Bulletin #2, ABI PRISM® 7700 Sequence Detector, PE Applied Biosystems, Foster City, CA). Briefly, the method involves obtaining the C_T values for the cytokine of interest, normalizing to a house-keeping gene (18 S in the present case), and deriving the fold increase compared to the control, unstimulated cells. Table 1 lists the primers sets used for these experiments. In preliminary experiments, the products were analyzed by gel electrophoresis, and a single product was obtained with each primer set. In addition, dissociation curves yielded single peaks.

In Vitro Experiments: All experiments were performed on pooled AM from several animals. AM were placed in 6 well plates, incubated for 2 h at 37^{o}C and washed to remove non-adherent cells. Then the cells were incubated with silica (200 μg/ml) or LPS (1 μg/ml) for 4 h for mRNA measurements, or 24 h for the measurement of inflammatory cytokines.

In Vivo Experiments: Rats were anesthetized with an intraperitoneal (i.p.) injection of 30-40 mg/kg body weight sodium methohexital (Brevital, Eli Lilly and Company, Indianapolis, IN) and were intratracheally instilled using a 20-gauge 4-inch ball-tipped animal feeding needle. Silica (Min-U-Sil 5) was suspended in endotoxin- and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS: BioWhittaker, Walkersville, MD), and rats received either 2 mg silica/100 g body weight or an equivalent volume of PBS. The

animals were sacrificed 4 h post-exposure and AM were isolated as described above. The lavaged lung tissue was used for isolation of total RNA.

Statistical Methods: A paired t-test was used for *in vitro* experiments. A t-test assuming unequal variance or a Z-test for means was used to evaluate the *in vivo* data. The significance was set at <0.05.

Results

Effects of Silica Treatment on Cell Viability: In initial experiments, the effect of silica treatment (200 μg/ml) on cell viability was assessed by measuring the release of LDH into the medium at the end of the 4 h incubation time. The numbers (U/L) for control vs. silica-treated cells (n = 3) were, for fibroblasts (49 ± 14 vs. 49 ± 15), for type II cells (87 ± 9 vs.91 ± 7), and for macrophages (101 ± 13 vs.100 ± 7).

Effects of Silica or LPS on mRNA Expression in AM in Vitro: AM were stimulated with either 200 μg/ml of silica or 1 μg/ml of LPS for 4 h. The expression of 10 genes, implicated in the induction of an inflammatory response, was measured by real-time RT/PCR. The message levels of only three cytokines (MCP-1, MIP-2, and IL-6) showed a significant increase at 4 h following *in vitro* exposure to silica (Figure 1). In contrast, mRNA levels for GM-CSF, ICAM-1, IL-1β, IL-10, iNOS, TGF-β1 and TNF-α were not significantly elevated following this treatment..

To compare the effect of silica with that of bacterial endotoxin, LPS, we also measured mRNA levels of these inflammatory mediators in AM stimulated with LPS for 4 h *in vitro* (Figure 2). LPS stimulation increased message levels of IL-1β, IL-6, GM-CSF, iNOS, MCP-1, MIP-2, and TNF-α but not those of ICAM-1, IL-10 and TGF-β1.

Effects of in Vivo Silica Treatment on mRNA Expression in Cells Obtained by BAL: Figure 3 shows the mRNA expression in cells isolated from rats, 4 h after intratracheal instillation of silica (2 mg/100 g body weight). The three cytokines that showed an increase at 4 h in vitro (IL-6, MCP-1 and MIP-2) also showed an increase in vivo. In addition, the expression of 4 other genes (GM-CSF, IL-1β, IL-10 and iNOS) was increased. Three genes (TGF-β1, TNF-α, and ICAM-1) showed no change either in vitro or in vivo.

Effects of Silica on mRNA Expression in the Lung Tissue Following Intratracheal Instillation: We also measured cytokine expression in the lavaged lung tissue 4 h following the intratracheal instillation of silica (Fig. 4). The results were similar for the most part as those seen in AM (Figure 3). There was a significant increase in the message levels of GM-CSF, IL-1β , IL-6, iNOS, MCP-1, MIP-2, and TNF-α. No increase was seen for ICAM-1, IL-10, and TGF-β1.

mRNA Expression of Cytokines in AM-Enriched and PMN-Enriched Fractions: One major difference between cells obtained by BAL from control rats vs. silica-treated rats is the presence of large number of PMNs in the silica-treated animals. One explanation for the differences seen in gene expression following *in vitro* and *in vivo* exposure may be

that the neutrophils produce additional cytokines, not seen with AM alone. To determine the role of PMN in mRNA expression following silica-treatment, we obtained AM-enriched and PMN-enriched fractions from BAL fluid of animals treated with silica *in vivo*. The mRNA levels were expressed in relation to the expression levels in relation to AM. It should be noted that these AM have been exposed to silica *in vivo* and express high levels mRNA as shown in figure 3, which was set as one in figure 5. Figure 5 shows that mRNA expression of the seven cytokines studied was essentially the same in the two fractions indicating that PMN enrichment is not the cause for differences between *in vitro* and *in vivo* treatments.

Cytokine/Chemokine Expression at the Protein Level: The levels of four cytokines/chemokines (IL-6, MCP-1, MIP-2, and TNF-α) were measured in the supernatants of AM cultures following 4 h incubation with either silica or LPS. There was no increase in the protein levels of these mediators with silica, but LPS produced very high levels of these cytokines/chemokines (Figure 6A-D). There was no increase in these mediators even after 24 h incubation with silica. In contrast, the cytokine levels of IL-6, MCP-1, and MIP-2 were increased in the alveolar lavage fluid when the animals were exposed to silica for 4h *in vivo* (Figure 7). There was no increase in TNF-α levels 4 h after exposure to silica either *in vitro* or *in vivo* (data not shown).

Co-culture of Type II Cells and AM on Gene Expression: In order to determine if the differences seen in mRNA expression in AM exposed to silica *in vitro* and *in vivo* may be related interaction between AM and type II cells we performed co-culture experiments. We focused on 4 genes which were upregulated only following *in vivo* exposure. Table 2

shows the expression of these 4 genes in co-cultures of AM and type II cells. Essentially, there was no difference in the expression of these genes when the cells were co-cultured with or without silica. These results indicate that the expression of these inflammatory mediators is not mediated by interaction between AM and type II cells.

Gene Expression in Lung Fibroblasts: Figure 8 shows the expression of 5 genes in fibroblasts cultured alone or in the presence of silica. We included IL-6 because lung tissue showed very high levels of IL-6 mRNA levels (Figure 4). The mRNA levels were expressed relative to mRNA levels of AM alone. It is clear that the mRNA levels for IL-6 and GM-CSF are very high in resting lung fibroblasts. IL-6 protein levels, as determined by ELISA, were a hundred fold higher in culture supernatants of lung fibroblasts compared to culture supernatants of AM (210 ± 75 vs. 2.4 ± 1.1, $n = 7$), indicating that the message is being translated into protein. In addition, in vitro exposure to silica caused a significant increase in mRNA levels, but this increase in mRNA levels was not reflected in an increase in protein synthesis (210 ± 75 vs. 231 ± 62, control vs. sililca, $n = 7$). This is similar to that seen in AM. We have not measured the GM-CSF protein levels.

Gene Expression in AM and Fibroblast Co-cultures: Table 3 shows the relative expression of the 5 genes in co-culture experiments. Although co-culture of AM and lung fibroblasts seems enhance iNOS, and IL-10 mRNA levels seen over and above that seen with each cell type alone, the results were extremely variable to draw a definitive conclusion concerning a synergistic effect. With regard to IL-6 and GM-CSF, the main

source seems to be fibroblasts but results were too variable to conclude whether co-cultures with or without silica enhance the mRNA levels.

mRNA Expression in AM and Lung Fibroblasts in Transwell Experiments (Table 4): The co-culture experiments do not allow determination of mRNA expression in individual cell types. Therefore, we conducted Transwell experiments to isolate RNA from each cell type and to study the roles of cell/cell contact vs. soluble mediators in these interactions. Two conclusions can be drawn from these experiments; first, that there is no difference in the mRNA levels of IL-1, IL-10 and iNOS under the different conditions tested. Second, the main source of IL-6 and GM-CSF are lung fibroblasts. Although AM seem to enhance IL-6 and GM-CSF mRNA levels in fibroblasts, the extreme variation in the results does not permit a definitive conclusion.

Combining the observations from co-culture experiments and Transwell experiments, it appears factors, in addition to cell/cell contact and soluble mediators secreted by these two cell types, are involved in regulating the inflammatory mediators in *in vivo* situation.

DISCUSSION

Exposure to silica causes inflammatory and fibrotic lung disease (Hnizdo and Vallyathan 2003). Silica- induced inflammatory response has been implicated in the pathogenesis of fibrosis. In this study, we measured expression of 10 genes which are

involved in regulating the inflammatory processes in the lung, at the message level. The studies were conducted in BAL cells and lung tissue following *in vivo* exposure; and in AM, Type II cells and lung fibroblasts following *in vitro* exposure.

Exposure of AM to silica *in vitro*, increased message levels of only 3 genes that included IL-6, MCP-1 and MIP-2. MCP-1 plays an important role in accumulation of monocytes (Leonard and Yoshimura 1990). MIP-2 is a potent chemotactic factor for neutrophils (Driscoll 1994). Upregulation of these two genes very early, following silica exposure, may account for the rapid accumulation of these cells in the lung. IL-6 is a pleiotropic cytokine with multiple biologic activities (Van Snick 1990) that has been shown to be upregulated following silica (Hetland et al. 2001) and asbestos (Simeonova et al. 1997) exposure of human lung epithelial cells. Here, we show that it is one of the early genes expressed in AM following silica exposure. Upregulation of these genes requires only the interaction between the silica particles and the macrophages.

When mRNA levels were measured in BAL cells harvested from rats instilled with silica it was found that the mRNA levels of 4 other genes (GM-CSF, IL-1, IL-10 and iNOS) went up in addition to the 3 genes mentioned above.

The production of NO in isolated AM from *in vivo* silica-treated animals and lack of NO production following *in vitro* treatment has been reported previously (Huffman et al. 1998). We confirm that observation. In order to determine if cell/cell interactions may be involved in the production of NO and the expression of three other genes

belonging to this group, AM macrophages were co-cultured with either type II cells or lung fibroblasts. The data (table 2) clearly indicate that co-culture with type II cells does not upregulate these genes under any of the conditions studied.

Co-culture of AM with fibroblasts showed that iNOS and IL-10 mRNA levels may go up, but the response was extremely variable and no definitive conclusions could be drawn. In addition, the Transwell experiments show that the co-culture of AM and fibroblasts does not significantly increase the message levels of GM-CSF, IL-1, IL-10 and iNOS levels in AM. Therefore, the factors responsible for upregulation of these genes following intratracheal instillation of silica remains elusive.

Three other genes (ICAM-1, TGF-1β, and TNF-α) did not show any change in BAL cells and lung tissue following *in vivo* treatment or in AM following *in vitro* treatment. The observation that the release of TNF-α is increased in the blood monocytes of miners with coal workers' pneumoconiosis (Borm et al. 1988)has led to several studies showing an increase in TNF-α levels from AM stimulated with silica (Baer et al. 1998; Dubois et al. 1989; Gossart et al. 1996). However, others have shown that *in vitro* treatment with silica does not induce TNF-α levels in human AM (Gosset et al. 1991). In some cases, where an increase in TNF-α production was shown, the levels were minimally increased and the levels were at least a couple of orders of magnitude less than what is seen with LPS stimulation (Kanj et al. 2002; Rojanasakul et al. 1999; Shi et al. 1999), raising the question of their biological relevance. In one *in vivo* study in rats with silica, an increase in mRNA levels of TNF-α in AM was not seen until 3 days after

intratracheal instillation (Gossart et al. 1996), and even later in a silica inhalation study (Porter et al. 2002). These data make the role of TNF-α in the initial stages of silica-induced inflammation questionable, even though TNF-α has been reported to be a key mediator in the eventual development of fibrosis (Piguet et al. 1990). In our studies, we did not observe any increase in mRNA in AM for TNF-α at 4 h *in vitro* and at 4 h *in vivo*. However, there was an increase in the mRNA expression in TNF-α in the lung tissue 4 h after intratracheal instillation of silica.

The cytokine, TGF-β1, and the adhesion molecule, ICAM-1, have also been implicated in the pathogenesis associated with silica exposure (Matrat et al. 1998; Nario and Hubbard 1996). We have not detected any increase in the message levels of these two genes either following *in vitro* or *in vivo* silica exposure. TGF-β1 is shown to be critical in acute lung injury (Pittet et al. 2001), but may not play a role in particle-induced lung disease, at least in the initial stages. ICAM-1 has been shown to be upregulated in LPS-induced lung inflammation (Madjdpour et al. 2000; Nathens et al. 1998). LPS stimulation *in vivo* has been shown to increase ICAM-1 expression both in AM (Grigg et al. 2003) and the lung tissue (Nathens et al. 1998). There was no increase in ICAM-1 message in AM after *in vitro* exposure to silica or LPS in the present study. A third gene which did not show any change with LPS was IL-10. Our findings are consistent with previous findings that there is no upregulation of TGF-β1 in AM (Xing et al. 1994) or IL-10 in lung tissue (Johnston et al. 1998) following LPS stimulation. With regard to ICAM-1, an increase was demonstrated in AM following *in vivo* exposure to LPS (Grigg et al. 2003). We evaluated ICAM-1 in AM macrophages after only *in vitro* exposure.

We observed significant increases in the mRNA levels of IL-6 and two chemokines (MCP-1 and MIP-2) at 4 h following *in vitro* treatment with silica. Although, the message levels showed an increase there was no increase in the protein levels measured in the supernatants of the cultures at 4 h. However, when silica was administered intratracheally, there was considerable increase in both message levels and protein levels at 4 h. Our findings with regard to the production of MCP-1 and MIP-2 are consistent with previous observations demonstrating an increase in these two chemokines following silica exposure (Driscoll et al. 1998; Driscoll 2000; Hubbard et al. 2002). The observation that the *in vitro* treatment upregulates the message levels without increasing the protein levels, but *in vivo* both message levels and protein levels go up, indicates that cell/cell interactions and/or other influences might play an important role in the expression of these cytokines at the protein level.

The Transwell experiments revealed that a major source of IL-6, GM-CSF in the lung could be lung fibroblasts. When mRNA levels were expressed relative to AM (table 4), the IL-6 levels in lung fibroblasts were several hundred fold higher than those in AM. Similarly, mRNA levels of GM-CSF were much higher in fibroblasts compared to AM. Further, the number of fibroblasts (interstitial cells) is 10 fold higher than AM in the lung tissue (Stone et al. 1992). These observations indicate that the fibroblasts are a major of source of these inflammatory mediators in the lung.

MCP-1 has significant involvement in the inflammatory disorders of the lung (Rose et al. 2003). It has been shown to regulate alveolar epithelial cell inhibition of fibroblast proliferation (Moore et al. 2002). In addition to monocytes, fibroblasts are an important source of MCP-1 (Galindo et al. 2001;Hao et al., 2003). We found MCP-1 mRNA levels were several fold higher in lung fibroblasts compared to AM (data not shown). Therefore, the main source of both IL-6 and MCP-1 in the BAL fluid following silica exposure could be lung fibroblasts. This is consistent with the observation that silica can directly stimulate lung fibroblasts (Arcangeli et al. 2001; Baroni et al. 2001). We have not evaluated the sources of MIP-2 in this study.

GM-CSF is purported to play an important role in numerous respiratory illnesses, including asthma (Xing et al. 1996). It is generated by a variety of lung cell types (Bergman et al. 2000; Blau et al. 1994; Christensen et al. 2001; Churchill et al. 1992; Fitzgerald et al. 2003; O'Brien et al. 1998; Smith et al. 1990; Soloperto et al. 1991; Trapnell and Whitsett 2002). GM-CSF was not produced by AM when stimulated with silica *in vitro*, but an increase in message levels were seen in both BAL cells and lung tissue following intratracheal instillation. This confirms the reported need for cell/cell interactions in the upregulation of GM-CSF (Fitzgerald et al. 2003).

The importance of cell/cell interactions in the production of inflammatory mediators has been emphasized in several studies. Direct contact between human peripheral blood mononuclear cells and renal fibroblasts facilitates the expression of MCP-1 (Hao et al. 2003). Similarly, macrophage/fibroblast interactions are important for

the production of GM-CSF (Fitzgerald et al. 2003). Both soluble mediators and adhesion molecules have been implicated in these interactions (Hao et al. 2003; Zickus et al. 2004). The lack of effect on the expression of several genes in co-culture experiments with contact or without contact (Transwell experiments) indicate that some additional factors may be involved in the regulation of cytokine production in the lung following silica exposure. It should be recognized that during inflammation that a variety of cells are recruited in to the lung and a number of products are generated. Any one of these factors may influence the expression of inflammatory mediators. In this regard it is important to keep in mind the role of lung surfactant. Lung surfactant is known modulate immune functions in the lung (Wright 1997); we mention its role in particular because we have some preliminary data to suggest that lung surfactant may enhance cytokine production in the lung fibroblasts.

In summary, we found that exposure of AM macrophages to silica *in vitro* upregulates only three genes (IL-6, MCP-1 and MIP-2). However, in BAL cells harvested following intratracheal instillation of silica 4 additional genes (IL-1, IL-10, iNOS and GM-CSF) were upregulated. Co-cultures of AM with alveolar epithelial type II cells or lung fibroblasts did not enhance mRNA level of the four additional genes that were expressed following *in vivo* exposure. There is need to evaluate the role of other mediators in regulating the production of inflammatory mediators in the lung, perhaps the role of lung surfactant. Most of the studies concerning silica-induced inflammatory processes in the lung have been focused on the role of AM, our Transwell studies show that lung fibroblasts are an important source of IL-6 and GM-CSF. These observations indicate that the fibroblast-derived inflammatory mediators may also play an important role following silica exposure.

Acknowledgments

We have no conflicts of interest to report.

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Figure Legends

Figure 1. mRNA expression in AM stimulated *in vitro* with silica (200 μg/ml) at 4 h post-exposure. The bars represent fold-increase above control (mean ± SEM of at least 4 experiments for each cytokine). *Significantly greater than control, $p < 0.05$.

Figure 2. mRNA expression in AM stimulated *in vitro* with LPS (1 μg/ml) at 4 h post-exposure. The bars represent fold-increase above control (mean ± SEM) in the message levels from a minimum of 4 different experiments with LPS. All bars were significantly different from control (*) except for ICAM-1, IL-10 and TGF-β1 (TGF).

Figure 3. mRNA expression in cells obtained by bronchoalveolar lavage from animals at 4 h following intratracheal instillation of silica (2 mg/100 g body weight). The bars represent fold-increase above control (mean ± SEM) in the message levels from a minimum of 5 different animals (in the control and treated groups). * Significantly greater than control, $p < 0.05$.

Figure 4. mRNA expression in the lavaged lung tissue isolated from animals at 4 h following intratracheal instillation of silica (2 mg/100 g body weight). The bars represent fold-increase above control (mean ± SEM) in the message levels from a minimum of 5 different animals (in the control and treated groups). * Significantly greater than control, $p < 0.05$.

Figure 5. mRNA expression in separated AM and PMN isolated from animals at 4 h following intratracheal instillation of silica (2 mg/100 g body weight). The bars represent fold-increase above control (mean ± SEM) in the message levels from 3 different animals.

Figure 6. Cytokine/chemokine protein levels as determined by ELISA in culture supernatants of AM treated *in vitro* with silica (200 μg/ml) or LPS (1 μg/ml) for 4 h, or silica for 24 h. There was no difference in IL-6 (A), MCP-1 (B), MIP-2 (C) and TNF-α (D) levels between control- and silica-treated cells at either exposure time. In contrast, LPS produced a large increase in all four cytokine/chemokines even as early as 4 h. Bars represent mean ± SEM from 4 separate experiments. * Significantly greater than control, $p < 0.05$.

Figure 7. Cytokine/chemokine protein levels as determined by ELISA in alveolar lavage fluid from animals at 4 h following the intratracheal instillation of silica (2 mg/100 g body weight). The bars represent mean ± SEM from 5 different animals (in the control and silica-treated groups). Silica treatment increased protein levels of the three mediators, IL-6, MCP-1 (A) and MIP-2 (B). * Significantly greater than control, $p < 0.05$.

Figure 8. mRNA expression in lung fibroblasts stimulated *in vitro* with silica (200 μg/ml) at 4 h post-exposure. The mRNA values were measured relative to that found in freshly isolated AM incubated for 4 h. The bars represent fold-increase

above control (mean ± SEM of at least 4 experiments for each inflammatory mediator). * Significantly greater than control, $p < 0.05$.

Table 1

Gene	Primers	Product (bp)
GM-CSF	Sense - GAC ATG CGT GCT CTG GAG AAC G Anti-sense - GCC ATT GAG TTT GGT GAG GTT GC	144
ICAM-1	Sense - AAT CTG ACC TGC AGC CGG AAA G Anti-sense – GGA GCT AAA GGC ACG GCA CTT G	108
IL-1β	Sense - AGC TCC ACG GGC AAG ACA TAG G Anti-sense - GGA TGG CTT CCA AGC CCT TGA C	155
IL-6	Sense - CCC AAC TTC CAA TGC TCT CCT AAT G Anti-sense - GCA CAC TAG GTT TGC CGA GTA GAC C	141
IL-10	Sense - GGC TCA GCA CTG CTA TGT TGC C Anti-sense - AGC ATG TGG GTC TGG CTG ACT G	116
iNOS	Sense - GTC ACC TAT CGC ACC CGA GAT G Anti-sense - GCC ACT GAC ACT CCG CAC AAA G	117
MCP-1	Sense - TCA CGC TTC TGG GCC TGT TG Anti-sense - CAG CCG ACT CAT TGG GAT CAT C	131
MIP-2	Sense - GGC AAG GCT AAC TGA CCT GGA AAG Anti-sense- CAC ATC AGG TAC GAT CCA GGC TTC	113
TGF-β1	Sense - GCT AAT GGT GGA CCG CAA CAA C Anti-sense- TGG CAC TGC TTC CCG AAT GTC	103
TNF-α	Sense - CGT CAG CCG ATT TGC CAT TTC Anti-sense - TGG GCT CAT ACC AGG GCT TGA G	116
18 S rRNA	Sense - GGA CCA GAG CGA AAG CAT TTG C Anti-sense - CGC CAG TCG GCA TCG TTT ATG	115

Table 2

Relative mRNA expression in alveolar macrophage (AM) and type II alveolar epithelial cells (Type II) co-cultures stimulated with silica (Si; 200 μg/ml) for 4 h [a]

	AM	AM + Si	Type II	Type II + Si	AM + Type II	AM + Type II + Si
IL-1β	1.47 ± .53	0.87 ± .34	0.61 ± 0.41	0.41 ± 0.27	1.10 ± 0.60	1.03 ± 0.49
IL-10	1.04 ± 0.13	0.30 ± 0.11	2.58 ± 1.01	5.74 ± 2.62	1.41 ± 1.46	2.21 ± 2.32
iNOS	1.04 ± 012	0.67 ± 0.24	0.49 ± 0.23	0.44 ± 0.16	1.75 ± 1.63	1.93 ± 1.47
GM-CSF	1.13 ± 0.27	1.00 ± 0.34	2.02 ± 1.19	3.88 ± 2.38	3.49 ± 0.71	5.01 ± 3.20

[a]The numbers represent mean ± SEM from at least three different experiments relative to mRNA levels in AM

Table 3

Relative mRNA expression in alveolar macrophage (AM) and lung fibroblasts (Fibro) co-cultures stimulated with silica (Si; 200 μg/ml) for 4 h [a]

	AM	AM + Si	Fibro.	Fibro.+ Si	AM + Fibro.	AM + Fibro.+ Si
IL-1β	1.03 ± 0.08	0.71 ± 0.12	0.08 ± 0.07	0.31 ± 0.29	1.49 ± 0.46	2.41 ± 0.91
IL-10	1.16 ± 0.11	1.04 ± 0.16	0.33 ± 0.17	0.78 ± 0.40	25.9 ± 15.8	26.8 ± 13.4
iNOS	1.14 ± 0.11	0.83 ± 0.19	0.58 ± 0.33	1.54 ± 0.93	8.22 ± 3.54	16.2 ± 9.95
GM-CSF	1.23 ± 0.21	1.06 ± 0.17	18.4 ± 7.0	58.9 ± 22.4	6.44 ± 2.4	58.0 ± 43.7

[a]The numbers represent mean ± SEM from at least three different experiments relative to mRNA levels in AM

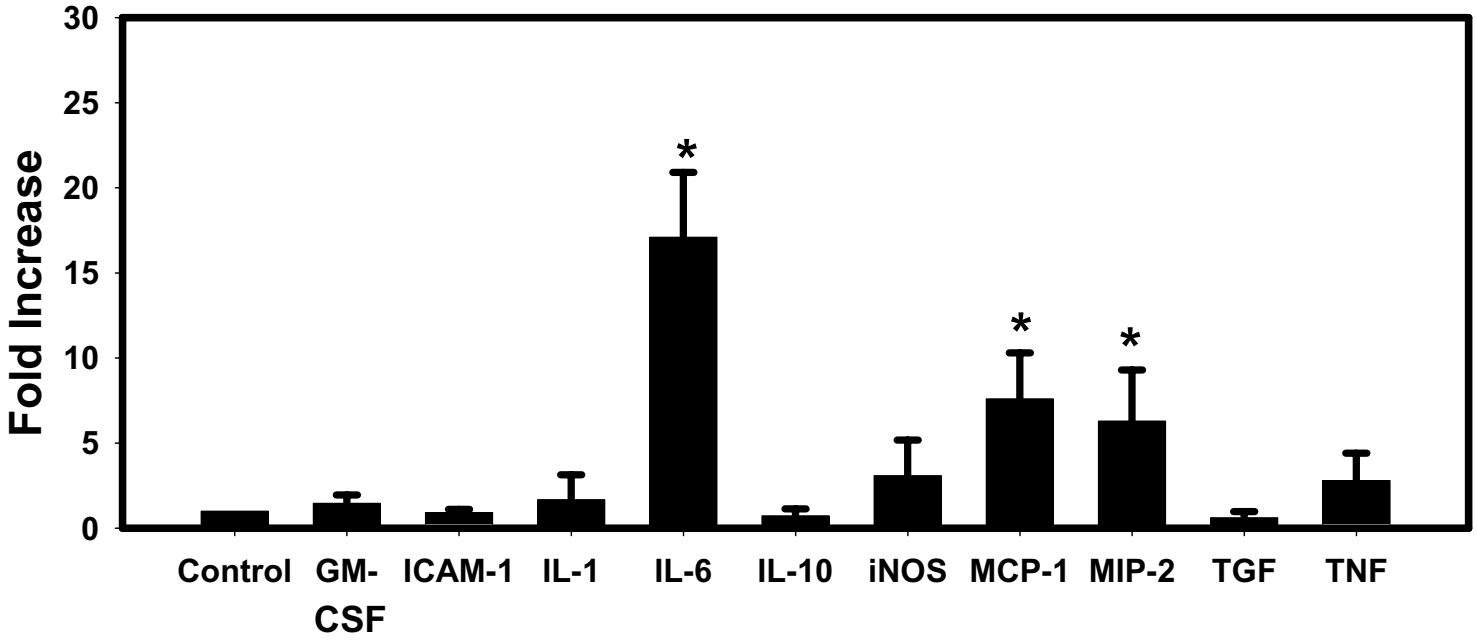
Table 4

Relative mRNA expression in alveolar macrophage (AM) and lung fibroblasts (Fibro.) stimulated with silica (Si; 200 μg/ml) for 4 h in Transwell experiments [a]

Insert	AM[b]	None	AM [b]	AM	AM [b] + Si	AM + Si	AM [b]	AM
Well	None	Fibro.[b]	Fibro.	Fibro.[b]	Fibro.	Fibro.[b]	Fibro.+ Si	Fibro. [b] + Si
IL-1β	1.0 ± 0.1	0.3 ± 0.2[1]	4.7 ± 2.5	3.1 ± 2.5	1.1 ± 0.5	0.6 ± 0.2	2.8 ± 2.1	1.0 ± 0.5
IL-6	0.7 ± 0.1	912 ± 565	1.1 ± 0.8	14487 ± 11702	16 ± 9.6	3009 ± 1344	4.8 ± 4.3	14573 ± 16524
IL-10	1.4 ± 0.4	1.6 ± 0.6	1.2 ± 0.6	3.5 ± 2.0	0.8 ± 0.5	1.3 ± 1.1	0.1 ± 0.1	0.7 ± 0.4
iNOS	0.8 ± 0.1	1.0 ± 0.1	2.6 ± 1.4	3.4 ± 1.6	3.9 ± 2.8	1.9 ± 1.5	1.5 ± 1.1	1.0 ± 0.4
GM-CSF	1.0 ± 0.3	4.2 ± 1.9	2.1 ± 0.7	41.6 ± 24	1.6 ± 0.9	42 ± 39	0.9 ± 1.0	19.3 ± 7.2

[a]The numbers represent mean ± SEM from three different experiments relative to mRNA levels in AM
[b] indicates the source of the cells in which the mRNA levels were measured.

Figure 1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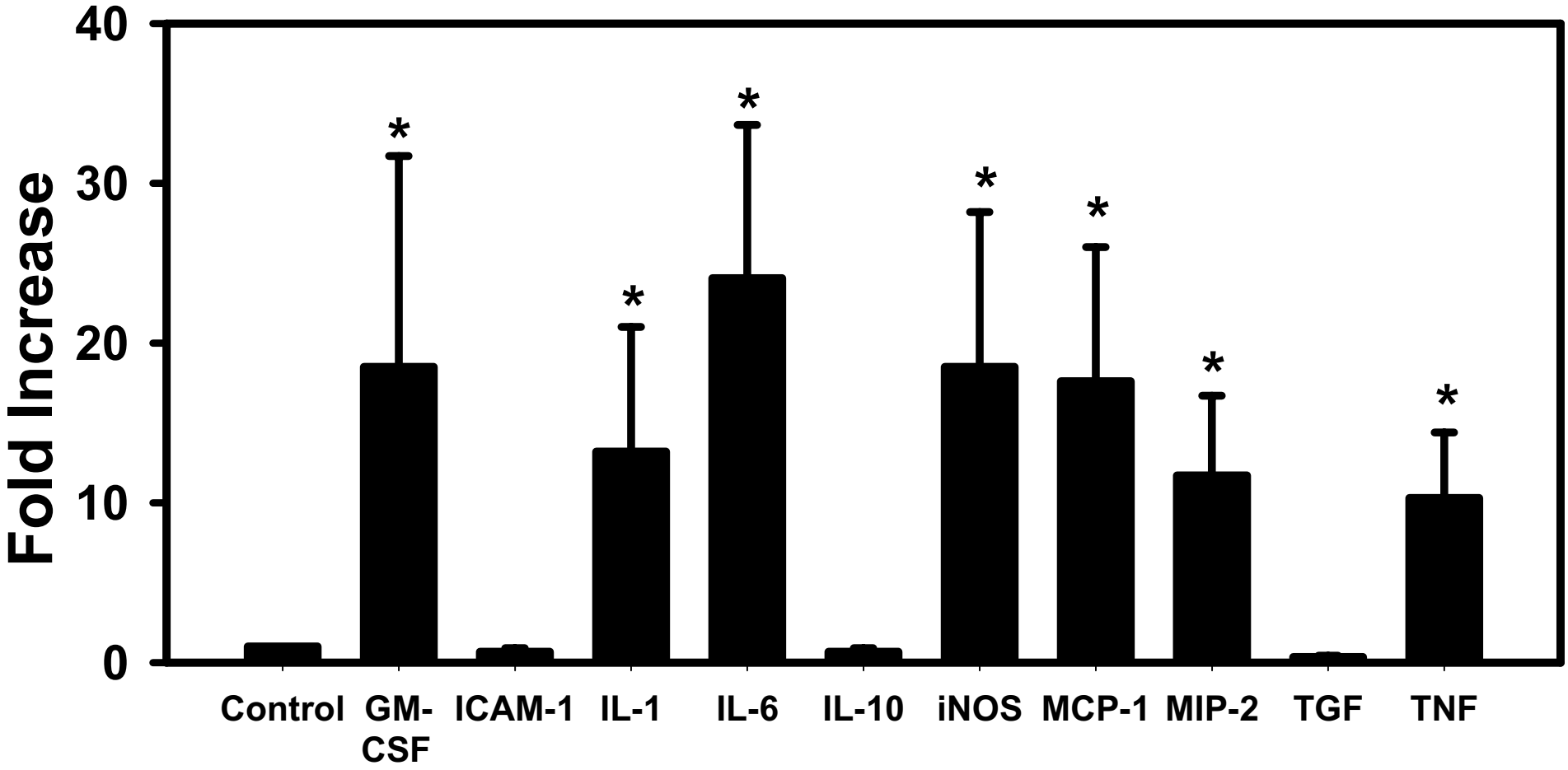
iNOS
MCP-1
MIP-2
TGF
TNF

Figure 2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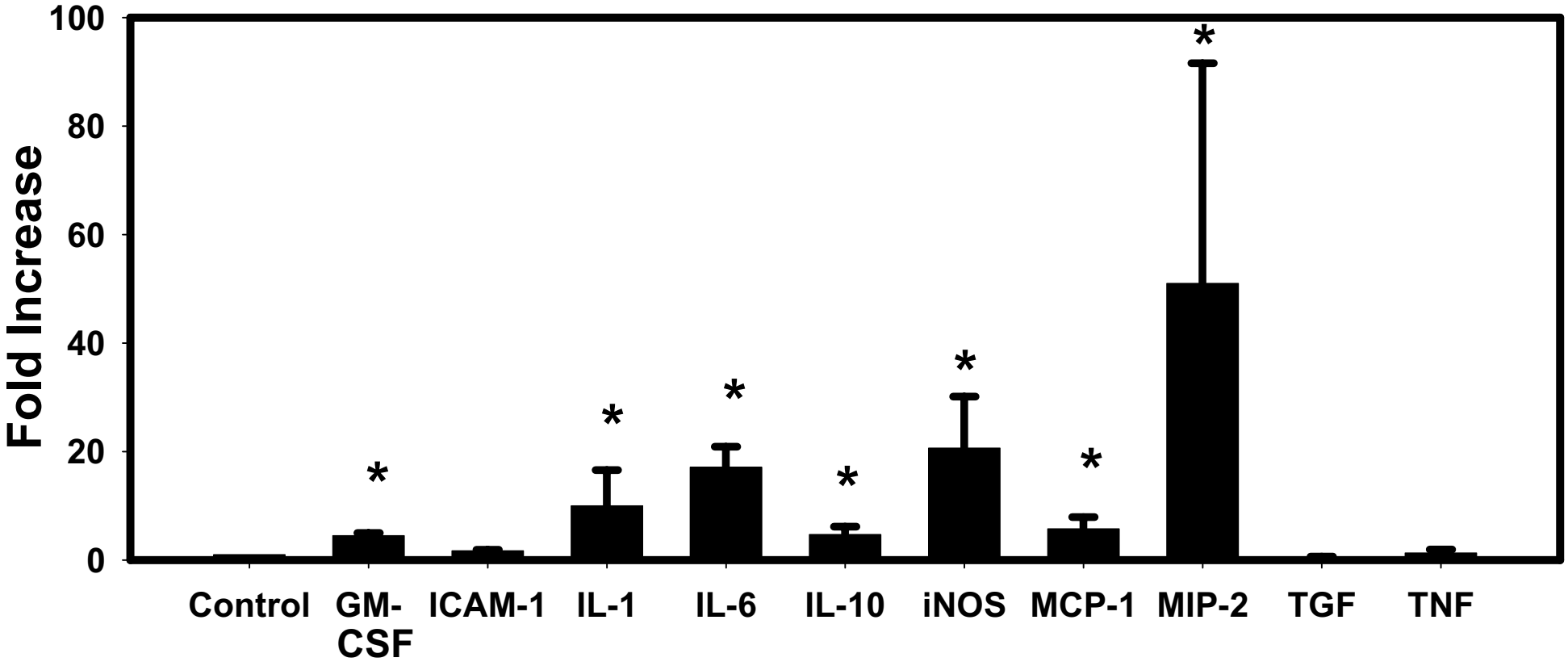
iNOS
MCP-1
MIP-2
TGF
TNF

Figure 3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Control
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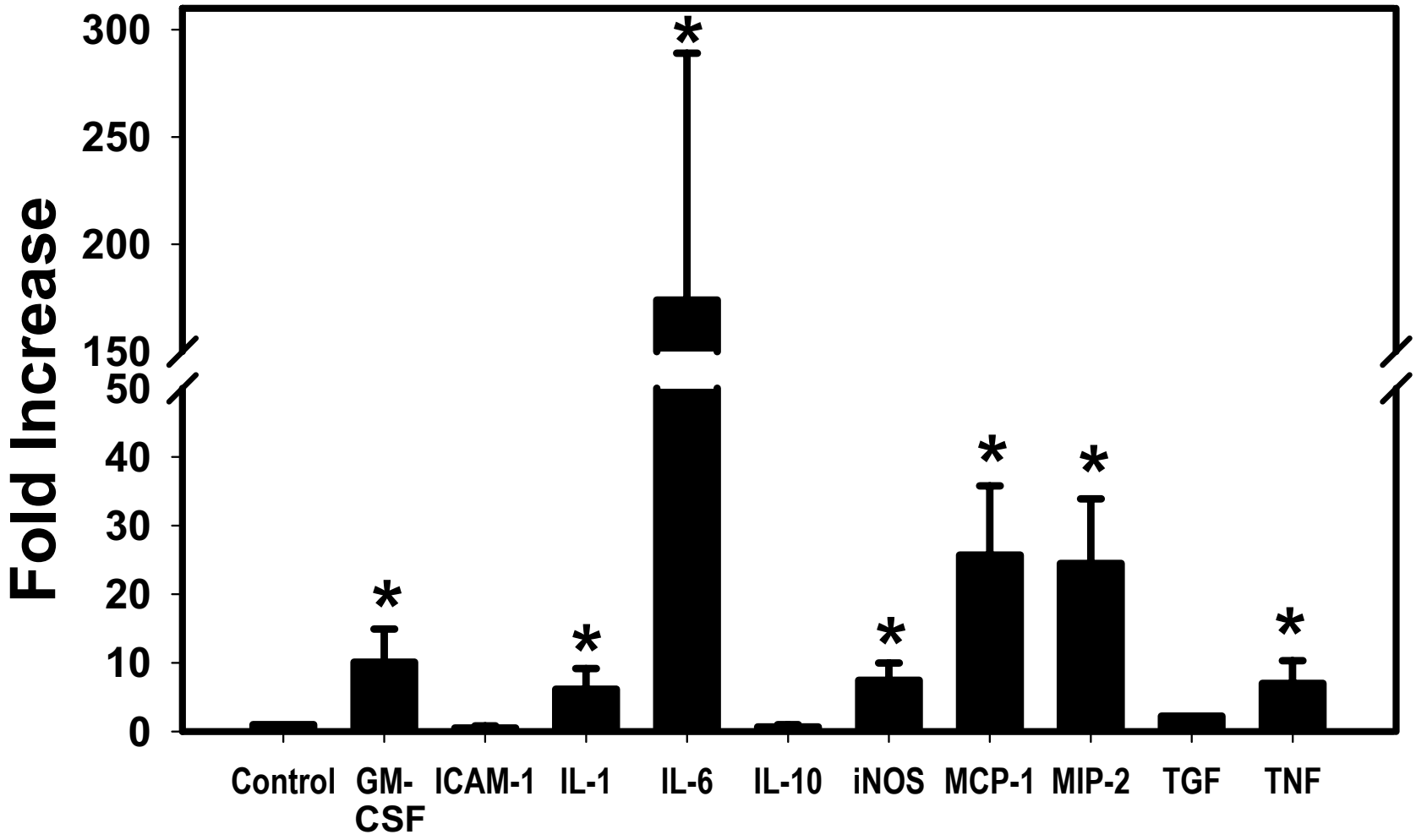
TGF
TNF

Figure 4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Control
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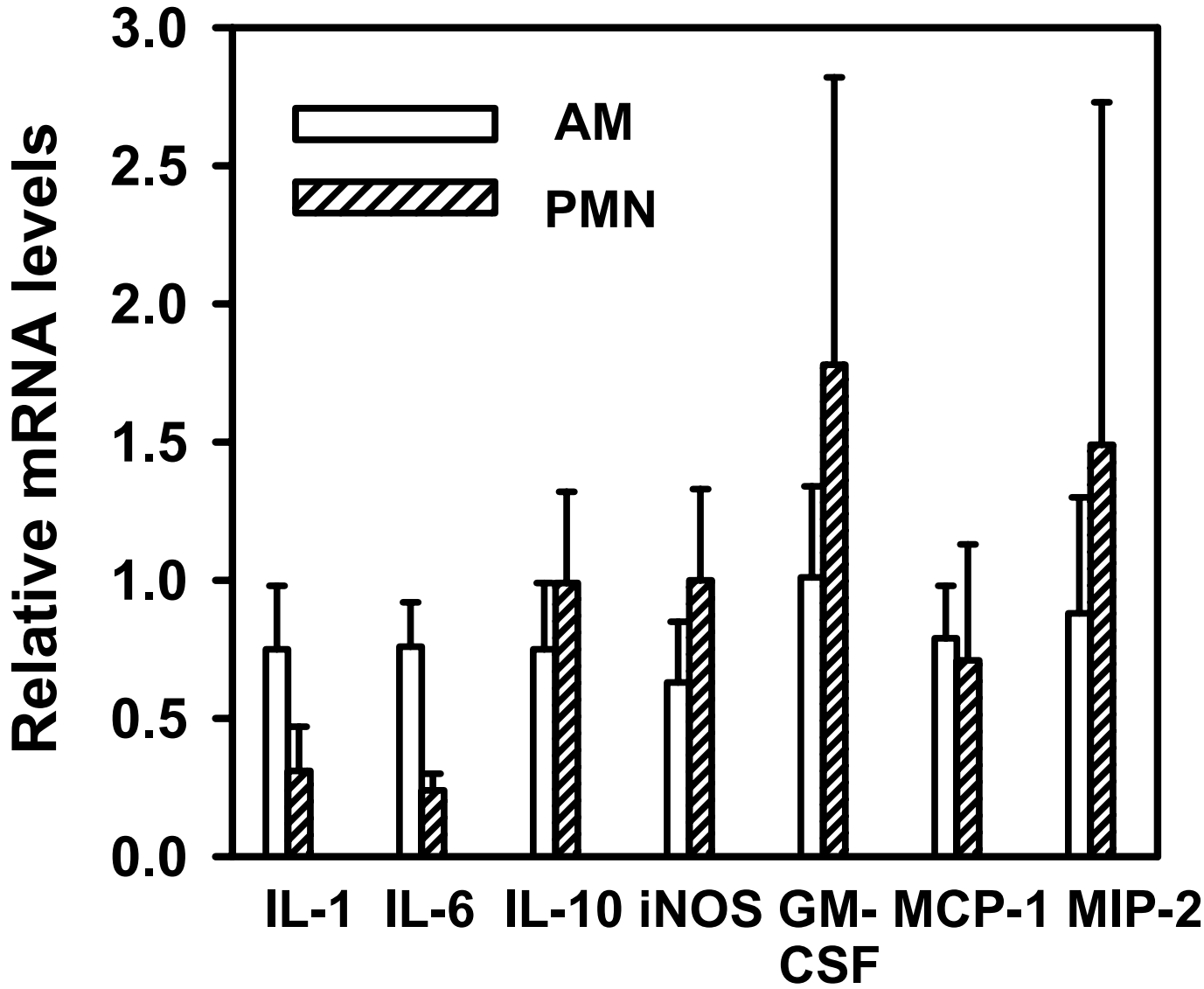
Figure 5


Relative mRNA levels
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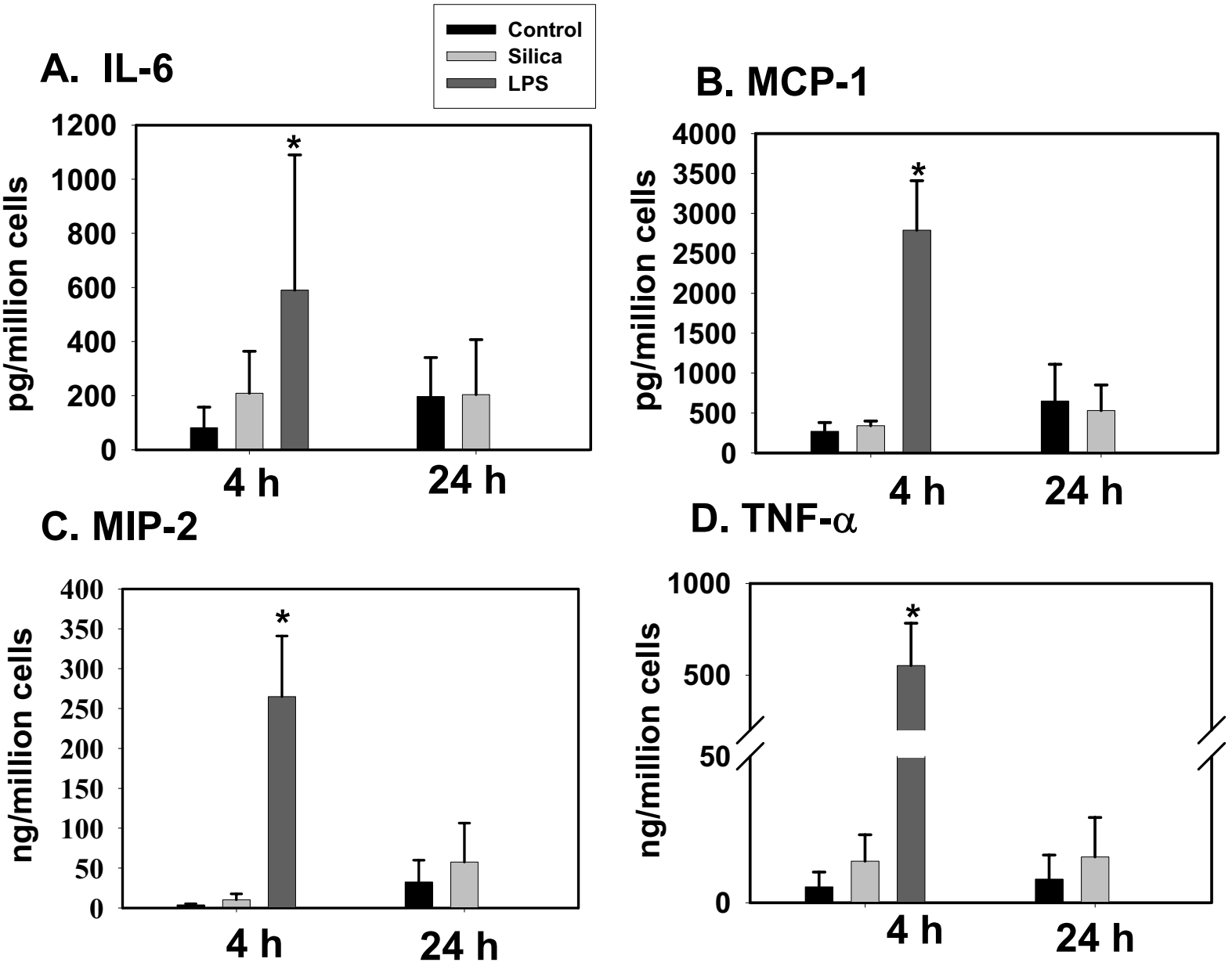
AM
PMN
IL-1
IL-6
IL-10
iNOS
GM-CSF
MCP-1
MIP-2

Figure 6


Control
Silica
LPS
A. IL-6
pg/million cells
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1000
800
600
400
200
0
*
4 h
24 h
B. MCP-1
pg/million cells
4000
3500
3000
2500
2000
1500
1000
500
0
*
4 h
24 h
C. MIP-2
ng/million cells
400
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100
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4 h
24 h
D. TNF-α
ng/million cells
1000
500
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4 h
24 h

Figure 7

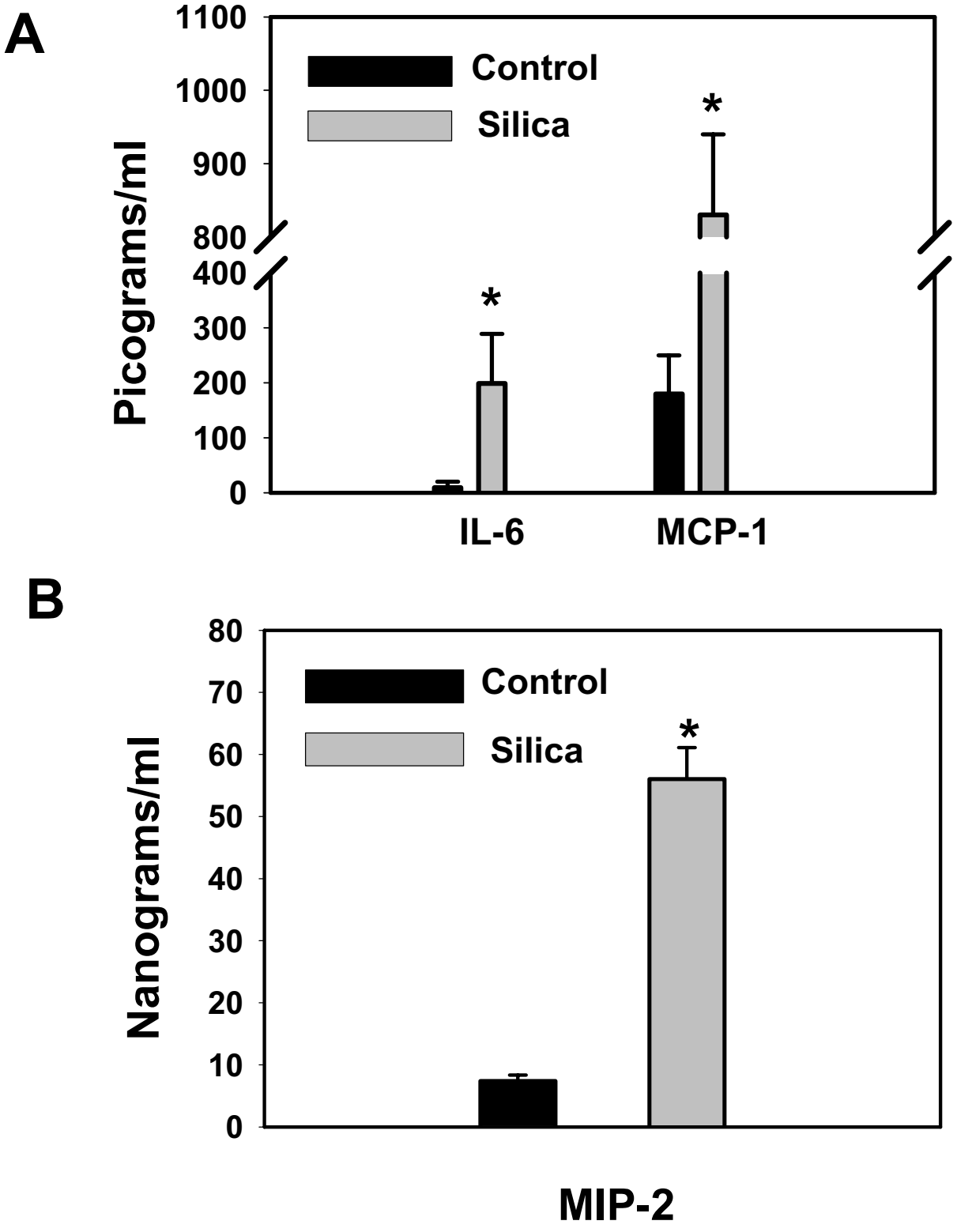
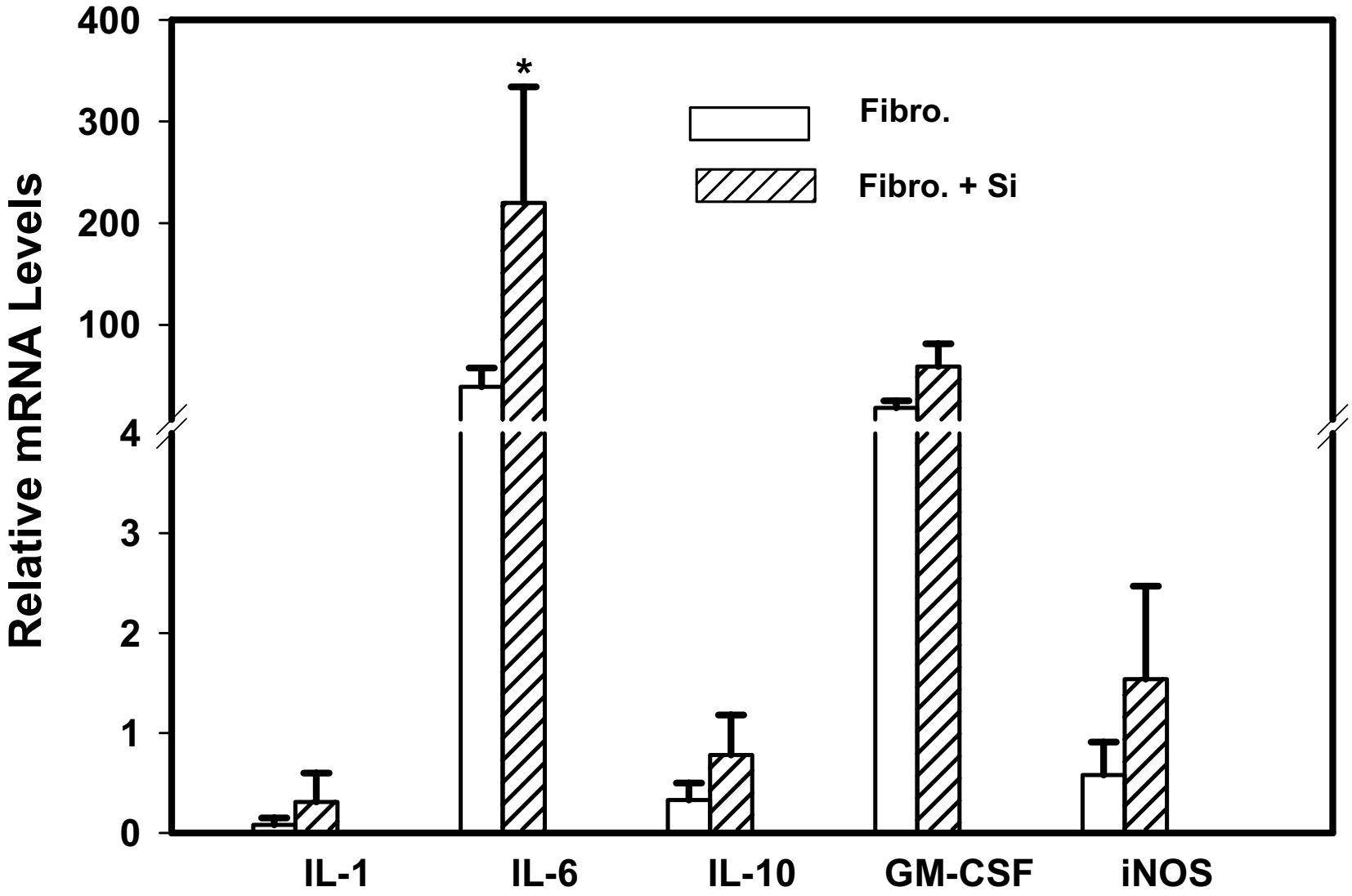

A
1100
1000
900
800
400
300
200
100
0
Picograms/ml
Control
Silica
*
*
IL-6
MCP-1
B
80
70
60
50
40
30
20
10
0
Nanograms/ml
Control
Silica
*
MIP-2

Figure 8


Relative mRNA Levels
400
300
200
100
4
3
2
1
0
*
Fibro.
Fibro. + Si
IL-1
IL-6
IL-10
GM-CSF
iNOS